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Hepatic biotransformation pathways and ruminal metabolic stability of the novel anthelmintic monepantel in sheep and cattle

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Monepantel (MNP) is a new amino-acetonitrile derivative anthelmintic drug used for the treatment of gastrointestinal (GI) nematodes in sheep. The present work investigated the main enzymatic pathways involved in the hepatic biotransformation of MNP in sheep and cattle. The metabolic stability in ruminal fluid of both the parent drug and its main metabolite (monepantel sulphone, $MNPSO_2$) was characterized as well. Additionally, the relative distribution of both anthelmintic molecules between the fluid and particulate phases of the ruminal content was studied. Liver microsomal fractions from six (6) rams and five (5) steers were incubated with a 40 μM of MNP. Heat pretreatment (50 °C for 2 min) of liver microsomes was performed for inactivation of the flavin-monooxygenase (FMO) system. Additionally, MNP was incubated in the presence of 4, 40, and 80 μM of methimazole (MTZ), a FMO inhibitor, or equimolar concentrations of piperonyl butoxide (PBx), a well-known general cytochrome P450 (CYP) inhibitor. In both ruminant species, $MNPSO_2$ was the main metabolite detected after MNP incubation with liver microsomes. The conversion rate of MNP into $MNPSO_2$ was fivefold higher ($P < 0.05$) in sheep (0.15 ± 0.08 nmol/min·mg) compared to cattle. In sheep, the relative involvement of both FMO and CYP systems (FMO/CYP) was 36/64. Virtually, only the CYP system appeared to be involved in the production of $MNPSO_2$ in cattle liver. Methimazole significantly reduced (41 to 79%) the rate of $MNPSO_2$ production in sheep liver microsomes whereas it did not inhibit MNP oxidation in cattle liver microsomes. On the other hand, PBx inhibited the production of $MNPSO_2$ in liver microsomes of both sheep (58 to 98%, in a dose-dependent manner) and cattle (almost 100%, independently of the PBx concentration added). The incubation of MNP and $MNPSO_2$ with ruminal contents of both species showed a high chemical stability without evident metabolism and/or degradation as well as an extensive degree of adsorption (83% to 90%) to the solid phase of the ruminal content. Overall, these results are a further contribution to the understanding of the metabolic fate of this anthelmintic drug in ruminants.

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INTRODUCTION

Anthelmintic resistance has become a global phenomenon in gastrointestinal (GI) nematodes of livestock (Kaplan & Vidyashankar, 2012). The discovery of new drugs to facilitate the management of resistance is a slow and costly process that results in few novel compounds being released (Woods & Knauer, 2010). The recently discovered amino-acetonitrile

derivatives (AADs) offer a new class of synthetic chemicals with anthelmintic activity (Kaminsky *et al.*, 2008). Monepantel (MNP) (ADD 1596), a representative compound of the AADs, was introduced into the veterinary pharmaceutical market for treating GI nematode infestations in sheep (Kaminsky *et al.*, 2008). After its oral administration, MNP is extensively metabolized and converted into different metabolites (Karadzovska *et al.*, 2009). However, the sulphone derivative ($MNPSO_2$), that exhibits a similar pharmacological activity against nematodes as the parent drug, is the main metabolite detected in plasma (Karadzovska *et al.*, 2009). High concentrations of MNP and $MNPSO_2$ are attained at specific gastrointestinal sites of parasite location in sheep (Lifschitz *et al.*, 2014).

Metabolic pathways involved in the hepatic biotransformation of MNP in sheep were qualitatively studied *in vitro* and *in vivo* (Stuchlíková *et al.*, 2013, 2014, 2016). The major outcome of these studies was the identification of several phase 1 and phase 2 metabolites formed in sheep liver. However, the main enzyme systems involved in the hepatic metabolism of MNP were not identified. As mentioned above, $MNPSO_2$ was found as the main oxidized metabolite of MNP formed in sheep liver. A two-step S-oxidation appears to be involved in the production of the sulphone metabolite (Karadzovska *et al.*, 2009; Stuchlíková *et al.*, 2013, 2014, 2016). Therefore, as S-oxidations are carried out by microsomal mixed function oxidases, both cytochrome P450 (CYP) and flavin-monooxygenase (FMO) systems may be involved in the production of $MNPSO_2$ in the liver of sheep and cattle. In this context, interspecies differences in the expression and function of hepatic xenobiotic metabolizing enzymes (XMEs) are well-documented (Nebbia *et al.*, 2003; Szotáková *et al.*, 2004; Gusson *et al.*, 2006) and may be relevant with regard to the hepatic metabolism of MNP. Considering that MNP would be launched to be used in cattle in the near future, the comparative metabolic pattern between sheep and cattle should be evaluated.

The GI tract of ruminant species constitutes an extra-hepatic compartment in which metabolic conversions are mediated by the digestive microflora (Prins, 1987). Anaerobic conditions predominate in the ruminal environment and reductive and hydrolytic reactions are of primary importance in the rumen. Therefore, the study of drug metabolic stability in this organ is particularly relevant when novel compounds are launched for enteral administration in ruminants. In addition, the degree of association of drugs orally administered with the particulate phase of the GI content may have a great impact on the pharmacokinetic behaviour of different compounds (Ali & Hennessy, 1996; Lifschitz *et al.*, 2005). Thus, the characterization of the adsorption of MNP and $MNPSO_2$ to the particulate material of the rumen digesta may contribute to understand the pharmacological features of this novel antiparasitic drug.

Based on the above-mentioned rationales, the present work investigated the comparative *in vitro* hepatic metabolism of MNP into its main (active) metabolite $MNPSO_2$ in sheep and cattle. The metabolic stability in ruminal fluid of both the parent drug and its sulphone metabolite was characterized as well. Additionally, the relative distribution of both anthelmintic molecules between the fluid and particulate phases of the ruminal content was studied.

MATERIALS AND METHODS

Reagents

Reference standards (99.5% pure) of MNP and its sulphone ($MNPSO_2$) metabolite were kindly provided by Novartis Animal Health Inc. (Basel, Switzerland). Stock solutions of each molecule (3 mg/mL) were prepared in acetonitrile (Baker Inc., Phillipsburg, NJ, USA). Working solutions were also diluted in acetonitrile. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Buenos Aires, Argentina). The solvents used for the chemical extraction and chromatographic analysis were HPLC grade (Baker Inc.). Buffer salts (KCl, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4 and CH_3COONH_4) were purchased from Baker Inc.

Animals and sample collection

Six (6) healthy Corriedale x Merino cross-breed rams (40–45 kg) were sacrificed to obtain samples of liver parenchyma for preparation of the microsomal fractions. Rams were fed with high quality lucerne hay and water *ad libitum*. Liver samples, free from pathological lesions, of five (5) Aberdeen Angus/Hereford cross-breed steers (300–350 kg) were obtained from a local slaughterhouse (Mirasur SA, Tandil, Argentina) located 16 km away from laboratory facilities. The animals were not subjected to any pharmacological treatment prior to slaughtering.

Animal procedures and management protocols were carried out according to the Animal Welfare Policy (Academic Council Resolution 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar) (Internal Protocol 12-2013). Animals were stunned and exsanguinated immediately in agreement with these institutional and internationally accepted animal welfare guidelines of the American Veterinary Medical Association (AVMA, 2013).

Preparation of subcellular fractions

In both species, the abdomen was opened immediately after sacrifice for liver ablation. Samples were rinsed with ice-cold KCl 1.15%, stored in aluminium foils and chilled in ice. Tissue samples were brought to the laboratory for subsequent procedures, which started within 1 h from sample collection and were carried out between 0 and 4 °C. The preparation of liver microsomes was performed as described previously (Maté *et al.*, 2008). Microsomal suspensions were stored at −70 °C until being used in the different biotransformation assays. An aliquot of each microsomal fraction was used to determine protein content using bovine serum albumin as a control standard (Lowry *et al.*, 1951).

Enzyme assays in liver microsomal fractions

Monepantel enzymatic biotransformation was assessed in liver microsomal fractions of sheep and cattle. The amount of $MNPSO_2$ formed in the presence of a NADPH-generating system was measured. A typical reaction mixture contained (in a final volume of 0.5 mL): phosphate buffer 0.1 M (pH 7.4), 0.5 mg of microsomal protein diluted in 100 μL of the same buffer, NADPH-generating system ($NADP^+$1.25 mM, glucose-6-phosphate 25.4 mM, $MgCl_2$ 5 mM, EDTA 0.8 mM and 6.25 U of glucose-6-phosphate dehydrogenase in phosphate buffer). Thawed microsomal suspensions were diluted in the phosphate buffer followed by the addition of the NADPH-generating system. The incubation mixtures were allowed to equilibrate (1 min at 37 °C) and the reaction started with the addition of MNP (40 μM). All incubations (30 min at 37 °C) were performed by duplicate in glass vials in an oscillating water bath under aerobic conditions. The final solvent concentration in the incubation was 2%. Blank samples, containing all components of the reaction mixture except the NADPH-generating system, were also incubated under the same conditions. All reactions were stopped by the addition of 0.4 mL of acetonitrile and stored at −20 °C until analysis.

The inactivation of the FMO system was performed by heating the diluted microsomal preparation (2 min at 50 °C) without NADPH and immediately chilling in ice (Dixit & Roche, 1984). Then, the NADPH-generating system was added and the reaction started with the addition of the substrate. The microsomal biotransformation of MNP was also studied in the presence of methimazole (MTZ, a FMO substrate/inhibitor) and pyperonil butoxide (PBx, a general CYP inhibitor). Microsomal preparations containing MTZ (4, 40 and 80 μM) or PBx (4, 40 and 80 μM) were pre-incubated (1 min at 37 °C) in the presence of NADPH and the reaction started with the addition of MNP (40 μM). Methimazole was dissolved in 10 μL of water, whereas PBx was dissolved in 10 μL of methanol (parallel control tubes contained the same volume of water or methanol).

Study of ruminal metabolism and stability

The ruminal metabolism of both MNP and $MNPSO_2$ was studied following the technique described by Virkel *et al.* (2002) and adapted by Lifschitz *et al.* (2005). Ruminal fluid samples were collected from three (3) rams and three (3) steers. Aliquots (2 mL) of sheep and cattle ruminal content were incubated with MNP or $MNPSO_2$ at 40 μM. Each incubation mixture was gently gassed with pure N_2 for 5 min at 38 °C. The final solvent concentration in the incubation was 0.5%. The incubations were carried out by triplicate in a shaking water bath at 38 °C under anaerobic conditions for 3, 6 and 24 h. After the incubation period, an aliquot of each sample (1 mL) was immediately frozen at −20 °C until analysis. Unfortified ruminal fluid samples ($n = 6$) were incubated and used as blanks. Control samples of boiled ruminal content ($n = 6$) were prepared and incubated under the same conditions to measure MNP and $MNPSO_2$ concentrations in the absence of active ruminal bacteria. In addition, inactivated samples of ruminal contents, spiked with MNP and $MNPSO_2$, were immediately extracted without incubation and used as controls ($n = 6$).

To establish the total partition of xenobiotics between fluid and solid contents, MNP and $MNPSO_2$ were incubated in the ruminal content during 3, 6 and 24 h as described above. Once the incubation period was over, the partitioning of MNP and $MNPSO_2$ between fluid and solid contents of ruminal digesta was determined following the technique described by Hennessy *et al.* (1994). Briefly, 1 mL of each incubation vial was centrifuged at 18 000 ***g*** for 15 min to separate the solid (particulate) and fluid phases of the ruminal content. Both solid and fluid contents were frozen at −20 °C until analysis. The current assay evaluated the formation of the drug/digesta complex in the rumen but did not establish the rate of partition.

Drug extraction and chromatographic analysis

The inactivated microsomal incubation mixture was centrifugated at 4000 ***g*** for 15 min at 4 °C. An aliquot (0.2 mL) of the supernatant was mixed with 0.3 mL of mobile phase (acetonitrile: methanol: water 60:8:32, v/v/v) and injected into the HPLC system. The extraction of MNP and $MNPSO_2$ from the ruminal fluid/particulate of sheep and cattle was carried out following the technique firstly described by Karadzovska *et al.* (2009) and adapted by Lifschitz *et al.* (2014). Briefly, the total ruminal content (1.0 mL), and the fluid and solid phases were mixed with 1 mL of acetonitrile. After mixing for 2 min, the solvent–sample mixture was centrifuged at 2000 ***g*** for 15 min. The supernatant was manually transferred into a tube, mixed with 5.0 mL of water and injected into a polymeric sorbent solid-phase extraction cartridge (Strata-X 33 lm Polymeric Sorbent 60 mg, Phenomenex, Torrance, CA, USA) conditioned with 1.0 mL acetonitrile and 1.0 mL water. The cartridge was washed with 2.0 mL of acetonitrile: water (40:60, v/v). Monepantel and $MPNSO_2$ were eluted with 1.5 mL of acetonitrile and concentrated to dryness under a stream of nitrogen. The resuspension was prepared with 250 μL of mobile phase (acetonitrile: methanol: water 60:8:32, v/v/v) and injected into the HPLC system. For MNP and $MNPSO_2$ quantification, 50 μL of each extracted sample was injected through an autosampler (Shimadzu 10 A HPLC system, Shimadzu Corporation, Kyoto, Japan). The HPLC analysis was performed using a reverse-phase C_{18} column (Kromasil, Eka Chemicals, Bohus, Sweden, 5 μm, 4.6 mm × 250 mm) and an acetonitrile: methanol: water 60:8:32, v/v/v mobile phase at a flow rate of 0.8 mL/min at 30 °C. Both analytes were measured by UV detection (SPD-10A; Shimadzu) reading at 230 nm.

Data analysis

Validation of the analytical procedures for extraction and quantification of MNP and $MNPSO_2$ was performed before starting the analysis of the experimental samples from the incubation trials. Known amounts of each analyte were added

to aliquots of boiled (inactivated) microsomal preparations and samples of ruminal content. The fortified samples were extracted and analyzed by HPLC (4 replicates) to obtain calibration curves, percentages of recovery, precision and accuracy. Calibration curves were prepared using the least squares linear regression analysis (Instat 3.00, GraphPad Software, Inc., San Diego, CA, USA) of HPLC peak area of analytes and nominal concentrations of spiked samples. The ranges of calibration curves were 0.05 to 5.0 nmol/mL (inactivated microsomes) and 0.4 to 32 nmol/mL (inactivated ruminal content). A lack-of-fit test was also carried out to confirm the linearity of the regression line of each analyte. The departure from linearity of the calibration curves was determined using ANOVA and run test (Instat 3.0; GraphPad Software, Inc.).

The concentrations in the experimental samples were determined following interpolation of peak area of the analytes into the standard curves. Absolute recoveries were established by comparison of the detector responses (peak areas) obtained for spiked microsomal or ruminal content samples and those standards prepared in mobile phase. The linear regression lines for MNP and $MNPSO_2$ for all matrices analyzed showed correlation coefficients $\geq$0.998. The absolute recovery percentage was >85%. The interassay precision and accuracy of the analytical procedures determined after HPLC analysis of MNP and $MNPSO_2$ showed a CV <20% and a relative error within 20%, respectively.

The reported data are expressed as mean ± SD. Metabolic rates are expressed in nmol of metabolic products formed per min per mg of microsomal protein (nmol/min·mg). Metabolic comparisons between species were performed by means of the Student *t*-test for unpaired observations (Instat 3.0; GraphPad Software, Inc.). The Student *t*-test for paired observations was used to compare data obtained after incubations carried out with control vs. heat-treated microsomal mixtures. ANOVA followed by Tukey multiple-range test was used to compare data obtained after incubations carried out in the presence of metabolic inhibitors. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Comparative hepatic biotransformation of MNP

Monepantel (40 μM) was incubated (30 min at 37 °C) with hepatic microsomal fractions of sheep and cattle. In both species, liver microsomes were able to convert the parent drug MNP into the sulphone derivative ($MNPSO_2$). However, significant differences were observed in the metabolic pattern of MNP between sheep and cattle liver microsomes. The metabolic rate of MNP in sheep liver microsomes was fivefold higher ($P < 0.05$) compared to that observed in the hepatic microsomal fraction of cattle. Metabolic rates of $MNPSO_2$ production in liver microsomes from both species are shown in Table 1. The relative involvement of FMO and CYP enzyme systems on MNP oxidation was estimated following incubations in heat-treated compared to control microsomal preparations from both species (Table 1). The FMO inactivation by heating produced a 32% inhibition ($P < 0.05$) on the rate of $MNPSO_2$ production in sheep liver microsomes. However, in cattle, the rate of production of $MNPSO_2$ did not change significantly after heat treatment of microsomal mixtures.

Figure 1 shows the effects of MTZ (FMO substrate/inhibitor) and PBx (CYP inhibitor) on the rate of $MNPSO_2$ production in liver microsomes from sheep and cattle. The presence of MTZ at different concentrations led to a significant reduction (between 41 and 79%, $P < 0.05$) in the rate of $MNPSO_2$ production in sheep liver microsomes. Conversely, MTZ did not inhibit MNP oxidation towards $MNPSO_2$ in cattle liver microsomes. On the other hand, PBx inhibited the production of $MNPSO_2$ in both sheep and cattle liver microsomes. In sheep liver microsomes, PBx significantly inhibited (between 58 and 98%, $P < 0.05$) the production of $MNPSO_2$ in a concentration-dependent manner. Conversely, in cattle liver microsomes, MNP oxidation was almost completely abolished by PBx, independently of the concentration of the inhibitor added to the incubation mixture.

Metabolic stability and relative distribution of MNP and $MNPSO_2$ in ruminal content

Both MNP and $MNPSO_2$ were metabolically stable in ruminal contents from sheep and cattle at the different incubation times assayed. Neither metabolic conversion nor chemical degradation was observed in ruminal contents from both species. The unchanged MNP and $MNPSO_2$ concentrations were between 85 and 122% of those observed in control incubations of sheep and cattle ruminant contents. Figure 2 shows the percentages of unchanged MNP and $MNPSO_2$ determined after their

Table 1. Relative involvement of flavin-monooxygenase (FMO) and cytochrome (CYP) systems on the hepatic oxidation of monepantel into monepantel sulphone ($MNPSO_2$)

	Metabolic rate of $MNPSO_2$ production			
	Control microsomes	Heat-treated microsomes[†]	FMO	CYP
Sheep	0.19 ± 0.09	0.13 ± 0.07**	0.06 ± 0.02 (32%)	0.13 ± 0.07 (68%)
Cattle	0.03 ± 0.01*	0.04 ± 0.01	–	0.03–0.04 (100%)

Metabolic rates are expressed in nmol/min·mg of microsomal protein. Mean (±SD) values were determined from 5 (cattle) or 6 (sheep) different liver microsomal preparations. [†]Heat treatment (2 min at 50 °C) of microsomal mixtures was performed for the inactivation of the FMO system (see Materials and Methods).

*$P < 0.05$ vs. sheep.

**$P < 0.05$ vs. control microsomes.

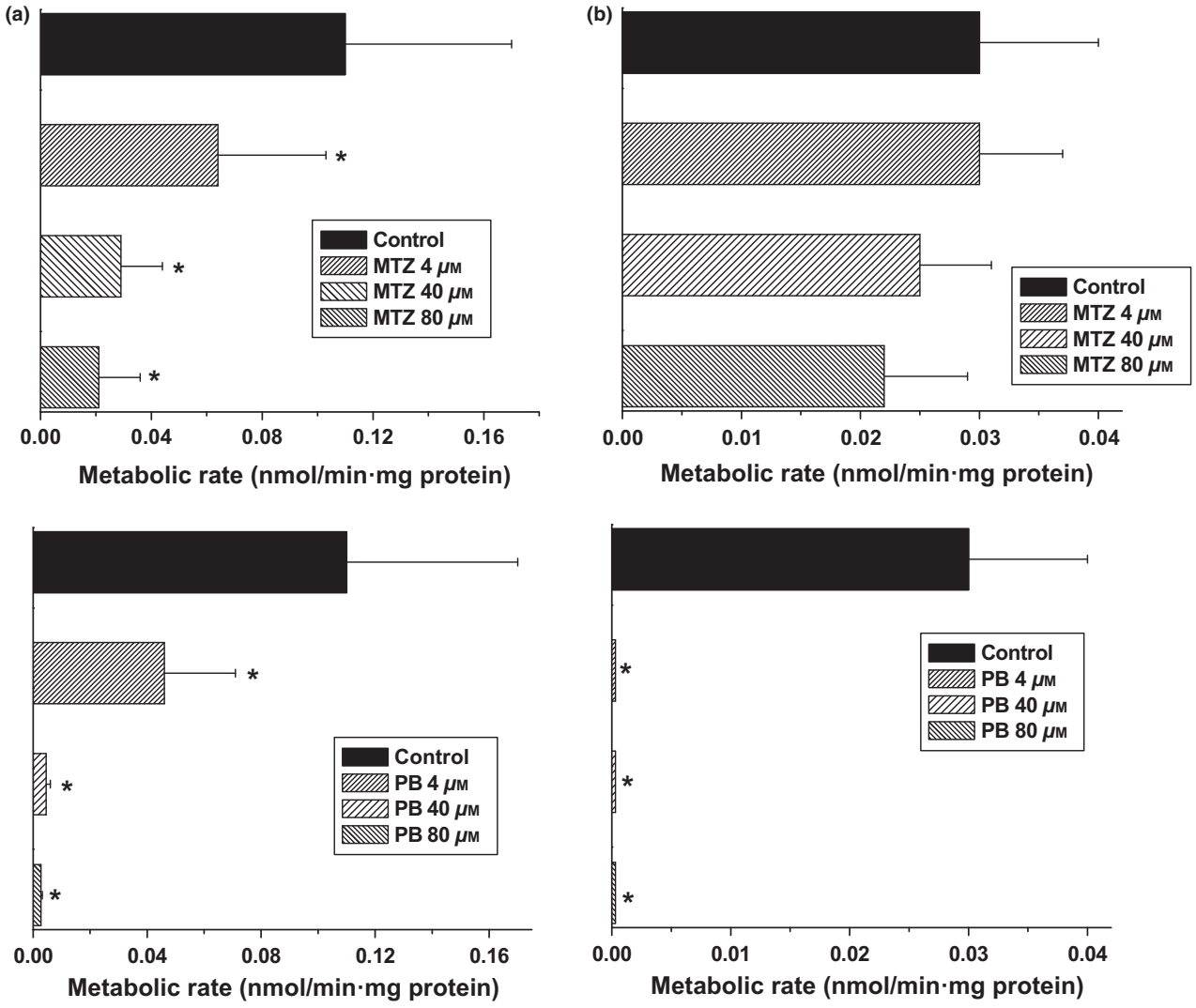

Fig. 1. Biotransformation of monepantel (MNP) by liver microsomal fractions collected from sheep (a) and cattle (b): Effects of different concentrations (4, 40 and 80 μM) of the metabolic inhibitors methimazole (MTZ) and piperonyl butoxide (PBx). The concentration of MNP was 40 μM. Metabolic rates of monepantel sulphone ($MNPSO_2$) production are expressed in nmol/min·mg of microsomal protein. Data (mean ± SD) were collected from 5 (cattle) or 6 (sheep) different liver microsomal preparations. *Values are statistically different ($P < 0.05$) vs. control incubations.

incubation in ruminal contents compared with those measured when both molecules were incubated in boiled (metabolically inactive) control samples. There was a singular partition of MNP and $MNPSO_2$ between solid and fluid phases of the ruminal content. In both species, significantly higher ($P < 0.05$) concentrations of the total analytes recovered (MNP: 92.2 to 93.8%; MNPSO2: 83.4 to 88.4%) were measured in solid (particulate) phase compared with the fluid phase of the ruminal content. Neither marked effect of the incubation time on the relative distribution of both molecules between phases was observed. No statistic differences in the percentage of drug bound to the particulate material were observed between MNP and $MNPSO_2$ at all incubation times with the ruminal content from both species. Figure 3 shows the partition of MNP between solid and fluid phases of ruminal contents from sheep and cattle.

DISCUSSION

The safety, efficacy and potential drug–drug interactions concerning the use of novel compounds in anthelmintic therapy require a detailed knowledge of drug metabolism in target species. Biotransformation of MNP has been studied in small ruminants both *in vitro* (Stuchlíková *et al.*, 2013, 2014) and *in vivo* (Karadzovska *et al.*, 2009; Stuchlíková *et al.*, 2014). Although it was demonstrated that MNP is metabolized into different derivatives and traces of them have been identified by a sensitive UHPLC/MS/MS analysis (Stuchlíková *et al.*, 2014), $MNPSO_2$ was found as the main pharmacological active metabolite detected after the oral administration of MNP to sheep, both in the bloodstream and in different tissues/fluids (Karadzovska *et al.*, 2009; Kinsella *et al.*, 2011; Lifschitz *et al.*, 2014). In sheep, the availability of $MNPSO_2$ in plasma was

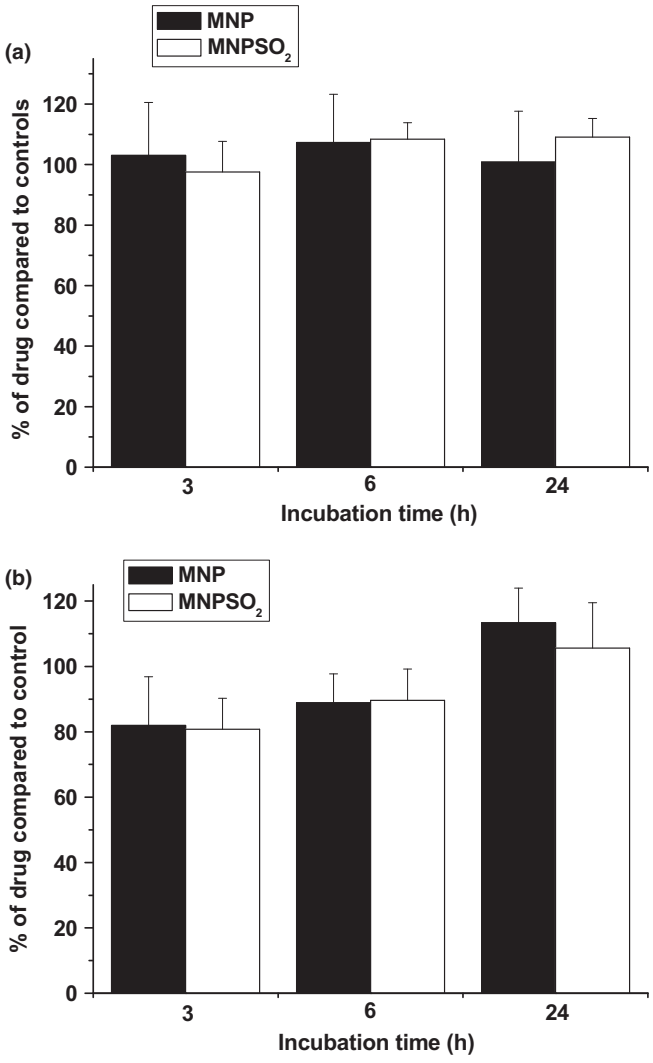

Fig. 2. Metabolic stability of monepantel (MNP) and monepantel sulphone ($MNPSO_2$) observed after their incubation (between 3 and 24 h) with sheep (a) and cattle (b) ruminal contents under anaerobic conditions. Data (mean ± SD, $n = 6$) are expressed as the percentage of unchanged drug/metabolite recovered from the incubation assays compared with those determined in the incubations with metabolically inactive (boiled) ruminal contents (control).

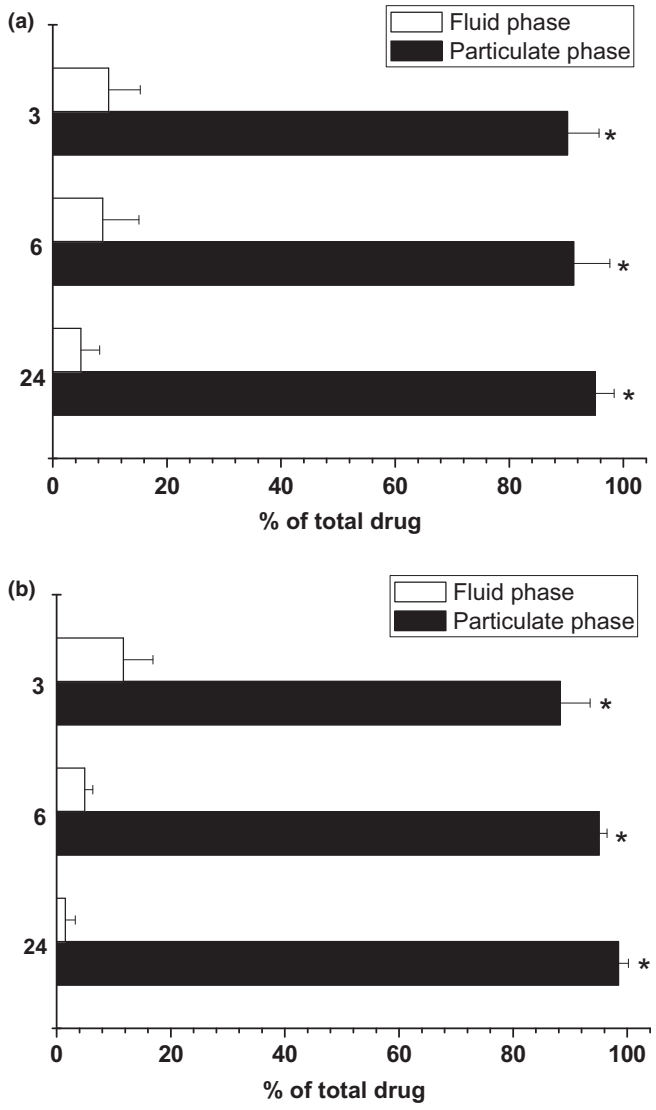

Fig. 3. Percentages (%) (mean ± SD, $n = 6$) of total monepantel (MNP) associated with the particulate (solid) and fluid phases of sheep (a) and cattle (b) ruminal contents after 3, 6 and 24 h of incubation. *Mean percentages of drug associated with the particulate phase are statistically different ($P < 0.05$) vs. those observed in the fluid phase.

found between 12- and 15-fold higher compared to MNP (Karadzovska *et al.*, 2009; Lifschitz *et al.*, 2014). Whilst the persistence of $MNPSO_2$ is significantly longer than MNP, the intermediate derivative, monepantel sulphoxide (MNPSO), is considered a short-lived metabolite (Karadzovska *et al.*, 2009). Overall, the efficient first-pass metabolism of MNP after its GI absorption may account for the early detection of $MNPSO_2$ in plasma (Karadzovska *et al.*, 2009).

The present work was addressed to gain further insight on the metabolic pathways involved on MNP biotransformation in the liver of sheep and cattle. As mentioned above, the metabolic conversion of MNP into $MNPSO_2$ also involves the production of an intermediate sulphoxide metabolite (Karadzovska *et al.*, 2009). It has been suggested that this sulphoxide metabolite is rapidly and almost completely converted into $MNPSO_2$. Consequently, the rate of appearance of the latter metabolite may also reflect the rate of production of the former. Compared to cattle, hepatic mixed function oxidases of sheep are more efficient to produce S-oxidations. For instance, the rate of production of albendazole sulphoxide (the main active metabolite of the anthelmintic albendazole) by sheep liver microsomes was significantly higher than that of cattle liver microsomes (Lanusse & Prichard, 1993). This rationale may also explain the higher oxidation rate of MNP (5-fold, see Table 1) observed in liver microsomes from sheep compared to cattle. This interspecies differences in the metabolic pattern of MNP should be considered whether this anthelmintic will be marketed for use in cattle.

Heat treatment of liver microsomes is a convenient method for the inactivation of the FMO enzyme system without

significant deleterious effects on the CYP-dependent metabolism (Grothusen *et al.*, 1996). Therefore, the relative participation of both enzyme systems may be estimated following the assumption that inactivation of FMO leaves the CYP system able to metabolize the substrate under study. In ruminants, the involvement of both CYP and FMO systems in the hepatic metabolism of sulphur-containing antiparasitic drugs (e.g.: albendazole, fenbendazole and triclabendazole) has been well documented (Virkel *et al.*, 2004, 2006). In the current work, both enzyme systems were found to be involved in the production of $MNPSO_2$ in sheep liver microsomes (see Table 1). However, the CYP-dependent metabolism may account for approximately 70% of the total S-oxidation of MNP in sheep liver. Besides, in cattle liver microsomes, $MNPSO_2$ was almost completely formed in a CYP-mediated reaction. The major involvement of the CYP system was confirmed when the metabolism of MNP was studied in presence of the metabolic inhibitors MTZ and PBx. In sheep liver microsomes, a higher inhibitory potency ($P < 0.05$) was observed for the CYP inhibitor (PBx) compared to the FMO inhibitor (MTZ) (see Fig. 1). However, in cattle liver microsomes, MTZ did not inhibit the metabolism of MNP, whereas the inhibitory potency of PBx was higher compared to that observed in sheep liver microsomes (see Fig. 1). Overall, these observations may confirm that MNP biotransformation in cattle liver depends, almost completely, on the CYP enzyme system.

As aforementioned, only the CYP system appeared to metabolize MNP in cattle liver. However, the hepatic CYP-dependent S-oxidation of MNP was higher (around 3.7-fold) in sheep compared to cattle (see Table 1). A survey of the available bibliography showed that hepatic CYP contents are between 0.64–0.91 nmol per mg of microsomal protein in sheep (Calléja *et al.*, 2000; Maté *et al.*, 2012) and 0.65–0.81 nmol/mg in cattle (Nebbia *et al.*, 2003; Giantin *et al.*, 2008). Based on these reported CYP contents in both species, it might be possible to estimate the CYP-dependent S-oxidation of MNP as turnover number (nmol/min·nmol of CYP). Thus, the hepatic CYP-dependent S-oxidation of MNP would be between 0.14–0.20 nmol/min·nmol of CYP in sheep and around 0.04–0.05 nmol/min·nmol of CYP in cattle. This rationale may confirm the relevance of the CYP-dependent metabolism of MNP in sheep (around 3.5-4-fold higher vs. cattle when is expressed as turnover number) and, clearly, may be a contribution to the understanding of interspecies differences in drug biotransformation. Further to these speculations, future research efforts should be addressed to the identification of specific isozymes involved in the metabolism of MNP in ruminants. In this regard, as FMO3 is the major flavin-containing isozyme expressed in sheep liver (Longin-Sauvageon *et al.*, 1998), it would be involved in the FMO-dependent S-oxidation of MNP in this species.

It is well-known that xenobiotic biotransformations take place predominantly in the liver. However, metabolic activity is apparent in extra-hepatic tissues (e.g.: intestinal mucosa, lung parenchyma and kidneys) and compartments such as the lumen of the GI tract. The GI microflora is very active in reductive reactions of foreign compounds. In this regard, the metabolic reduction of xenobiotics containing nitro and sulphoxide groups is particularly important in the rumen of sheep and cattle. Biodegradation of ronidazole (Vynckier & Debackere, 1993) and nitroxinil (Irazoqui *et al.*, 2014) by ruminal bacteria contributed to their poor systemic oral bioavailability. On the other hand, the active metabolites of the anthelmintics albendazole and fenbendazole, namely albendazole sulphoxide and oxfendazole, are reduced back to their parent (and more potent) drugs (Virkel *et al.*, 2002). However, the metabolic reduction of their respective sulphones was not observed in ruminal fluids of sheep and cattle (Lanusse *et al.*, 1992). The results of the current work are in agreement with these earlier observations, since $MNPSO_2$ was metabolically stable in ruminal contents of both ruminant species. On the other hand, as anaerobic conditions predominate in the ruminal environment, substrates are only partially oxidised. This fact may explain why neither MNP nor other sulphur-containing moieties, such as albendazole or fenbendazole, are oxidized in extent in the rumen's lumen.

The GI disposition of different anthelmintic drugs given orally to ruminants may be influenced by their pharmaceutical dosage form, concomitant drug treatments and several host-related factors. The degree of adsorption to the particulate material of GI contents has been among the most studied factors effecting the gastrointestinal disposition of benzimidazole anthelmintics in sheep and cattle (Taylor *et al.*, 1992; Lanusse & Prichard, 1993; Hennessy *et al.*, 1994; Sánchez *et al.*, 1997). Likewise, the association of orally administered anthelmintics to the particulate phase of the GI content was early described for oxfendazole (Ali & Hennessy, 1995), ivermectin (Steel, 1993; Ali & Hennessy, 1996; Lifschitz *et al.*, 2005) and moxidectin (Lifschitz *et al.*, 2005). Similar percentages of adsorption to the particulate phase of ruminal contents were determined for MNP and $MNPSO_2$ in sheep and cattle. The percentage of adsorption to the solid material observed in the current work was similar to those previously reported for the macrocyclic lactones ivermectin and moxidectin, which showed values ranging between 90.8 and 98.6%, respectively (Lifschitz *et al.*, 2005). Earlier investigations demonstrated that the rumen acts as a reservoir and prolong the duration of absorption and/or outflow down the GI tract of different benzimidazole anthelmintics (reviewed in Hennessy, 1993; Lanusse & Prichard, 1993). This well-known ruminal 'reservoir effect' mostly depends on the extent of adsorption of drugs/metabolites to the particulate phase of ruminal contents. This rationale may explain the prolonged GI disposition of MNP, compared to that observed in the systemic circulation, when this anthelmintic was orally given to sheep (Lifschitz *et al.*, 2014).

In conclusion, $MNPSO_2$ was the main metabolite formed after MNP incubation with liver microsomal fractions of sheep and cattle. Interspecies differences were observed in MNP S-oxidation; a higher rate of production of MNPSO2 was observed in liver microsomes of sheep compared to cattle. Both FMO and CYP enzyme systems were found to be involved in the

production of $MNPSO_2$ in sheep liver microsomes. However, this metabolic reaction seems to be almost completely produced by the CYP system in cattle liver. Both MNP and $MNPSO_2$ were metabolically stable in ruminal fluid contents from sheep and cattle. Furthermore, they were extensively associated with the solid phase of the ruminal contents of both ruminant species. These results may be a further contribution to the understanding of the kinetic behaviour of this novel anthelmintic drug in livestock.

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